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Bridging Ral GTPase to Rho Pathways

RLIP76, A Ral EFFECTOR WITH CDC42/Rac GTPase-ACTIVATING PROTEIN ACTIVITY*

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RalA and RalB are GTPases of unknown function and are activated by proteins, RalGDS, that interact with the active form of another GTPase, Ras. To elucidate Ral function, we have searched for proteins interacting with an activated form of RalA using the two-hybrid method and a Jurkat cell library. We have identified a partial cDNA encoding a protein, RLIP1, which binds to activated RalA and this binding requires an intact effector domain of RalA. Biochemical data with purified RalA confirm the genetic results. This protein also bears a region of homology with GTPase-activating protein (GAP) domains that are involved in the regulation of GTPases of the Rho family and, indeed, RLIP1 displays a GAP activity acting upon Rac1 and CDC42, but not RhoA. This GAP region is not required for RLIP1 binding to Ral.

The whole cDNA was cloned, and it encodes a 76-kDa polypeptide, RLIP76, which also binds RalA. The Rho pathway is involved in membrane and cytoskeleton modifications after mitogenic stimulation and acts in parallel to and synergistically with the Ras pathway. We propose that these pathways are linked through a cascade composed of Ras → RalGDS → Ral → RLIP76 → CDC42/Rac1/Rho, allowing modulation of the Rho pathway by the Ras pathway.

Ral proteins are biochemically well characterized GTPases whose functions have long remained elusive (1, 2). A potential clue was provided by the finding that RalGDS[1] and a RalGDS-like protein, which are activators of RalA and RalB (3), interacts with the activated form of Ras and that this interaction requires the integrity of the Ras effector domain (4–6). Thus RalGDS, and therefore Ral proteins, might be involved in transducing pathways that signal through Ras.

In order to decipher Ral function, we have searched for proteins that interact with the activated form of RalA. Using a two-hybrid method and a mutant of RalA deficient in its intrinsic GTPase activity (RalAV23), we have isolated a partial cDNA encoding a protein (RLIP1, Ral interacting protein 1) that has characteristics of a Ral effector protein.

The whole cDNA was isolated and sequenced; it contains an ORF encoding a predicted 76-kDa protein (RLIP76) that binds RalA.

Out of the Ral binding region, RLIP76 contains a GAP region related to RhoGAP domains and this structural homology reflects a functional homology with a GAP activity acting upon CDC42HS and Rac1.

EXPERIMENTAL PROCEDURES

Two-hybrid Screen—The two-hybrid system and the Jurkat cells library used in this study have already been described (7–9). For LexA fusion protein expression, we have used plasmids pBTM116 or derivatives with modified polylinkers. When necessary, PCR using *Pfu* polymerase was performed to generate adequate cloning sites. The bait of our screen was a fusion between LexA and a Val-23 mutant of RalA (equivalent to RasV12) deleted of its 27 C-terminal amino acids (RalAV23ΔCT) supposed to be involved in post-translational modifications and membrane localization. This fusion protein was expressed in yeast from plasmid pLRTA. LexA fusion proteins expression was checked on Western blots with anti-LexA antibodies (a gift from P. Moreau, Gif sur Yvette, France).

Yeast and the two-hybrid procedures were handled according to published methods (9, 10). Library plasmids from transformed yeast colonies were recovered using HB101 as a recipient strain, selected on M9 medium lacking leucine. When mating was used for two-hybrid tests; strain L40 was mated with strain AMR70 (*MATα, leu2, trp1, his3, ade2, URA3::lexAop-lacZ*) (a gift from S. Fields). When two-hybrid results are presented, we are showing the results of β-galactosidase test on filter paper. There was no discrepancy between the His auxotrophy test and the β-galactosidase test.

When required, point mutations were introduced using the Transformer site-directed mutagenesis kit (Clontech). Any DNA fragment submitted to mutagenesis and all PCR products were sequenced.

Gene Expression—Gene expression was analyzed on a multiple tissue Northern blot (Clontech) where mRNAs from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas were represented. A β-actin cDNA was used as a control.

cDNA Cloning—5′-RACE was carried out using 5′-RACE-ready cDNA from Clontech and following manufacturer's instructions. Isolation of cDNA from phage was performed according to usual techniques (11).

In Vitro Transcription/Translation of RLIP1—A PCR reaction was carried out with pRLIP1 as a template and adequate primers. The 5′ primer contains a T3 RNA polymerase recognition site upstream of an ATG initiation codon in frame with RLIP1 (CGAATTAACCCTCACTAAAGAAGGATGGAGATCCTAGAACTAGTCGG) (12). The 3′ primer is downstream of the stop codon of the amplified fragment (GTAAAAC-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L42542.

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[1] The abbreviations used are: GDS, guanine nucleotide dissociation stimulator; aa, amino acid(s); GAP, GTPase-activating protein; MBP, maltose-binding protein; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; FISH, fluorescence *in situ* hybridization.

GACGGCCAG).

Transcription and translation in presence of [^{35}S]methionine were performed using 1 μg of PCR product, and, sequentially, a mRNA capping kit and an *in vitro* translation kit (Stratagene).

Preparation of Proteins from Escherichia coli and in Vitro Binding Experiments—GST and of GST-Ral proteins from *E. coli* transformed with native or recombinant plasmid pGEX-4T1 were prepared following classical methods. All buffers contained 5 mM $MgCl_2$. For *in vitro* binding studies, 5 μg of glutathione-Sepharose 4B-bound proteins were washed twice in ice-cold binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM Pefabloc, and 0.5% Nonidet P-40) and incubated overnight at 4 °C with 1 μl of *in vitro* translated ^{35}S-RLIP1 in 50 μl of binding buffer containing 0.5 mM GTP (experiments with GST and GST-RalAV23), or GDP (in the cases of GST-Ral). After sedimentation of the beads, the supernatant ("the unbound fraction") was removed and the beads were washed three times with binding buffer containing 1 mM dithiothreitol. "Bound proteins" were recovered by boiling the beads in sample buffer. Unbound and bound fractions were subjected to SDS-PAGE on a 10% acrylamide gel. After staining with Coomassie Blue to detect the GST and GST-Ral proteins, gels were treated with Amplify (Amersham Corp.) and dried, and the presence of ^{35}S-RLIP1 was detected by autoradiography.

In vitro binding studies after guanine nucleotide exchange were performed as described above with the following alterations; 10 μg of glutathione-Sepharose 4B-bound proteins were washed twice in ice-cold exchange buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Pefabloc), and incubated for 3 h at 4 °C in 50 μl of exchange buffer containing 90 μM GTP or GDP. The nucleotide exchange reaction was stopped by adding $MgCl_2$ to 20 mM followed by two washes with binding buffer containing 20 mM $MgCl_2$.

In all cases, protein concentrations were estimated by Coomassie Blue staining of SDS-PAGE gels and adjustments were made for the same amount to be used in all experiments.

Protein Purification and GTP Hydrolysis Assay—RLIP1 was expressed as a MBP fusion protein from vector pMal-c2 (New England Biolabs). Rac1 and Bcr-GAP proteins were expressed in *E. coli* and purified as GST fusion proteins, then clipped off with thrombin. CDC42 was a gift from P. Boquet. [γ-^{32}P]GTP-bound Rac1, CDC42, and Rap2A were prepared by incubating 200 nM protein with 25 mM Tris, pH 7.5, 5 mM EDTA, 0.2 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 10 mM [γ-^{32}P]GTP (2 mCi to 30 Ci/mmol, DuPont NEN) in a 50-μl volume for 15 min at room temperature. GTP hydrolysis was initiated by raising $MgCl_2$ and GTP to final concentrations of 20 mM and 200 μM, respectively. GTP hydrolysis was stopped at different time points by addition of 2 ml of 50 mM ice-cold Tris, pH 8, 35 mM $MgCl_2$, 1 mM dithiothreitol, 150 mM NaCl, then quantitated by rapid vacuum filtration on BA 85 nitrocellulose filter and radioactivity counting (derived from Ref. 13). The GTP hydrolysis was conducted as described above, but in the presence or absence of Bcr-GAP (400 nM), or MBP-RLIP1 (800 nM), and stopped after a 10-min incubation at room temperature.

FISH Analysis—Fluorescence *in situ* hybridization (FISH) to metaphase chromosomes prepared from a normal male was carried out according to an usual technique (14). The probe was a 1.6-kilobase pair DNA fragment from one of the inserts isolated during the two-hybrid screen.

Sequence Analysis—We used a Sequenase sequencing kit (Amersham Corp.) or a deaza-T7-sequencing kit (Pharmacia Biotech Inc.) for classical sequencing, or a Dye-deoxy terminator kit (Perkin Elmer) and an Applied Biosystems model 373A automatic sequencer. Sequence analysis was performed using computer facilities provided by the Centre Interuniversitaire de Traitement Informatique (CITI2) (15).

RESULTS

Two-hybrid Screen—Around 1,000,000 colonies were screened with RalAV23ΔCT as a "bait" and a Jurkat cells library. Two library plasmids, pRLIP1 and pRLIP2, were recovered that contained partial cDNAs expressing proteins RLIP1 and RLIP2 (for RRal interacting protein) fused to GAL4 activation domain, respectively. RLIP1 and RLIP2 interact specifically with RalAV23ΔCT, as opposed to several irrelevant proteins (lamin, stathmin (Ref. 16), hSos1 (Ref. 17)) (data not shown). RLIP2 is a C-terminal part of RLIP1.

Ral allele dependence of the interaction was checked using pRLIP1, RalAV23, RalAV23A46 (an effector domain mutant), RalAA26 (which mimics a Ras GDP-blocked mutant), and

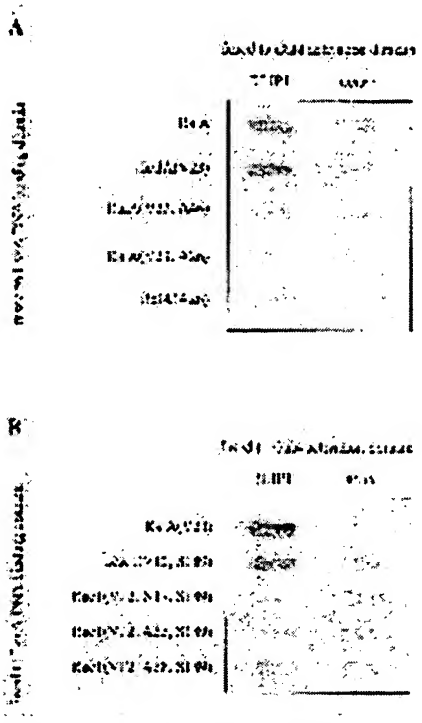

FIG. 1. **Allele-specific interaction of RalA and Rac1 with RLIP1.** L40 yeast cells were transformed with pairwise combinations of plasmids expressing proteins fused to LexA from a TRP1 plasmid and proteins fused to the activation domain of Gal4 (*GAL AD*) from a LEU2 plasmid. Transformed cells were patched on selective medium (DO-WL), then replicated on a Whatman No. 40 paper laid on a DO-WL plate. After 24 h, a β-galactosidase activity assay on paper was performed. In *A* and *B*, alleles of RalA and of Rac1 were expressed as LexA fusion proteins, respectively. Western blot analysis has shown that Ral alleles are expressed at a similar level, as are Rac alleles (data not shown).

RalAV23A26 were cloned in pBTM116. Fig. 1*A* shows the signals displayed in a β-galactosidase test. First, RLIP1 is able to interact not only with RalAV23ΔCT (data not shown) but also with RalAV23 and RalAwt. Second, RLIP1 interaction with RalAA26 or with RalAV23A26 is undetectable. Since a G26A mutation is supposed to block RalA in a GDP-bound state, this result suggests that RLIP1 binds to RalA only when this latter is bound to GTP and not to GDP. Third, RLIP1 is unable to interact with a RalAA46 mutant. Based on sequence and structure similarities with c-Ha-Ras, a T46A RalA mutant would have an impaired effector domain. This result suggests that RalA requires an intact effector domain to bind to RLIP1. It also suggests that, in yeast, LexA-Ralwt is, at least in part, in the GTP-bound conformation, as is the case for other GTPases expressed as LexA fusions (9).

From these data, it emerges that RLIP1 is a good candidate to be an "effector" of RalA function.

In Vitro Binding—In order to confirm the genetic data, we have tested *in vitro* RLIP1 binding to GST-RalA and GST-RalAV23 proteins prepared from *E. coli*. Around 30% of both RalA proteins bind GDP and GTP (data not shown). RLIP1 cDNA was amplified by PCR. The PCR product was transcribed from a T3 promoter sequence included at the 5′ end of the 5′ PCR primer, and translated *in vitro*. A ^{35}S-labeled protein of apparent molecular mass of 66 kDa was produced (Fig. 2*A*). Fig. 2*B* shows that RLIP1 did not bind to GST or to GST-RalA but does bind to GST-RalAV23.

When *in vitro* guanine nucleotide exchange was performed prior to RLIP1 binding, RLIP1 again did not bind to GST. It did

not bind GST-RalA or GST-RalAV23 loaded with GDP. It bound GST-RalA and, even better, GST-RalAV23 loaded with GTP (Fig. 2*C*). These data show that RLIP1-RalA interaction is not mediated by a yeast protein and are consistent with the genetic results, *i.e.* RLIP1 interacts directly with the GTP-bound form of RalA whose effector domain is required.

RLIP1 Binds Ral but No Other GTPase except Rac—We addressed the question whether RLIP1 discriminates among GTPases. Table I summarizes the results obtained with the two-hybrid method, using different GTPases and pRLIP1. It also gives the positive controls used in each case.

In the Ras superfamily, within the Ras branch (to which RalA belongs; Ref. 18), RLIP1 was not able to interact with c-Ha-Ras or with Rap1A, Rap2A, or Rap2B. However, it does interact with RalB.

In the Rab branch, RLIP1 was not able to interact with Rab5, Rab6, Rab7, or Rab13.

Finally, in the Rho/Rac branch, we were not able to detect any interaction with RhoA, RhoB or RhoG, but RLIP1 is able to interact with Rac1 (Fig. 1*B*).

This latter interaction was further investigated using Rac1 alleles. Fig. 1*B* shows that RLIP1 is able to interact with Rac1V12S189 but not with Rac1V12N17S189, a dominant negative mutant blocked in the GDP-bound form, or Rac1V12A35S189 and Rac1V12A38S189, two effector domain mutants (19). These results suggest that Rac1 bound to GTP is able to interact with RLIP1 through its effector domain.

The GAP-like Region Displays a GAP Activity—Sequence analysis of RLIP1 (see below) has revealed a region highly homologous to GAP regions acting upon GTPases of the Rho family. The question whether the region of RLIP that looks like a GAP region is a GAP was addressed. RLIP1 was expressed fused to MBP from plasmid pMAL-c2. The fused protein was purified and assayed for stimulation of the GTPase activity of purified CDC42 and Rac1, and Rap2A as a control. Fig. 3 shows that there is no effect on Rap2A (specificity control), a significant and reproducible effect on Rac1 and a stronger effect upon CDC42. It also shows that this effect is weaker that the one obtained with Bcr-GAP protein (positive control) (20). RLIP1 exhibited no GAP activity upon RhoA (data not shown).

Molecular Biology of the cDNA—Northern blot analysis revealed that RLIP1 is expressed in all tested tissues as a 4-kilobase mRNA of low abundance (data not shown).

Two consecutive rounds of 5′-RACE were required to obtain the full-length cDNA that was also recovered from a skeletal muscle cDNA library in λgt10 and from a placenta cDNA library in λEXlox. The sequence of the full-length cDNA was

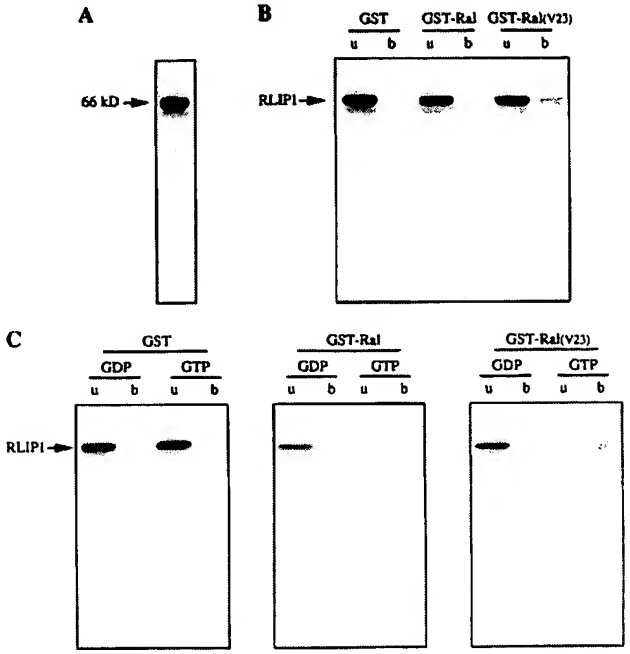

FIG. 2. **RalA and RLIP1 interact *in vitro*.** *A*, *in vitro* synthesized ^{35}S-RLIP1 was run on a SDS-PAGE (10% acrylamide) gel. Autoradiography shows that RLIP1 migrates as a 66-kDa protein. *B* and *C*, equal amounts of ^{35}S-RLIP were incubated with glutathione-Sepharose beads bound to equal amounts of GST, GST-RalA, or GST-RalAV23, preloaded (*C*) or not (*B*) with GDP or GTP (see "Experimental Procedures"). After overnight binding at 4 °C, beads were centrifuged and the supernatant removed. Beads were washed three times and boiled in SDS sample buffer. Half of the beads (the bound fraction, *b*) and half of the supernatant (the unbound fraction, *u*) were analyzed by SDS-PAGE followed by autoradiography.

TABLE I
Interaction of RLIP1 with different GTPases

L40 was cotransformed with pairwise combinations of plasmids, one expressing the GTPases listed above as LexA-fusion proteins, the other expressing RLIP1 from plasmid pRLIP1 isolated during our two-hybrid screen for RalA interacting proteins. Column "Protein expression" refers to verification of expression of the fused protein by Western blotting. This verification was carried out during this work or by the referred person. ND, not determined. Column "Control by two-hybrid assay" means that the construct used to express the LexA-GTPase fusion protein has permitted to detect a specific partner using L40 and a two-hybrid assay. Usually this positive control was assessed by the referred person who was the kind donator of the plasmid.

GTPase	Interaction with pRLIP1	Protein expression	Control by two-hybrid assay	Reference
RalA	+	+	+	This work
RalB	+	+	+	This work
cHRas	−	ND	+	(9)
cHRas(V12)	−	ND	+	(9)
Rap1A	−	ND	+	J. de Gunzburg, personal communication
Rap2A	−	+ (ref)	+	J. de Gunzburg, personal communication
Rap2A(V12)	−	+ (ref)	+	J. de Gunzburg, personal communication
Rab5	−	+	+	M. Zerial, personal communication
Rab6	−	ND	+	(34)
Rab5(L79)	−	+ (ref)	+	M. Zerial, personal communication
Rab6(L72)	−	+ (ref)	+	(34)
Rab7(L67)	−	+ (ref)	ND	P. Chavrier, personal communication
Rab13	−	+ (ref)	+	A. Zahraoui, personal communication
RhoA	−	+ (ref)	+	B. Olofsson, personal communication
RhoB	−	+	+	B. Olofsson, personal communication
RhoB(V14)	−	+ (ref)	+	B. Olofsson, personal communication
Rac1(V12S189)	+	+	+	This work; J. Camonis and G. Gacon, unpublished data
RhoG(V12ΔCT)	−	+	+	P. Fort, personal communication

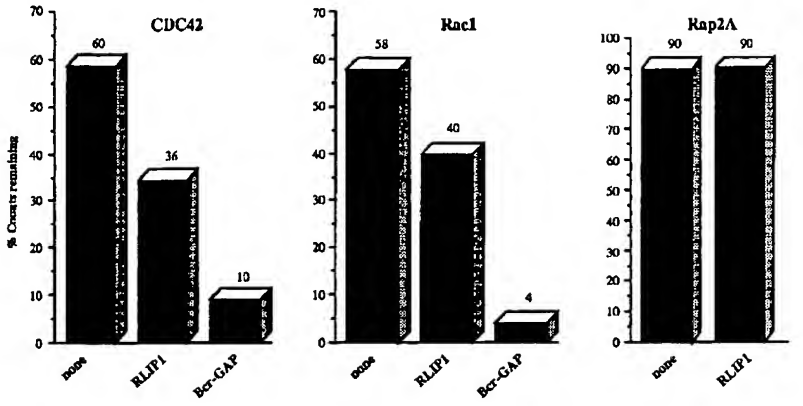

FIG. 3. **GTPase activating activity of RLIP1 upon Rac1 and CDC42.** Purified Rac1, CDC42, and Rap2A proteins were loaded with [γ-^{32}P]GTP, and GTPase activity was assessed by a filter binding assay (30). 100% refers to the radioactivity bound to the protein at time 0. For each protein, the *first column* reflects the intrinsic GTPase activity, the *second* the GTPase activity in presence of RLIP1. For CDC42 and Rac1, the *third column* reflects the GTPase activity in presence of the GAP region of Bcr, which harbors a powerful GAP activity upon Rac1 and CDC42.

A Sequence of RLIP76

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M T E C F L P P T S S P S E H R R V E H G S G L T R T P S S   30
E E I S P T R F P G L Y R T G E P S P P H D I L H E P P D V   60
V S D D E K D H G K K K G K F K K K E K R T E G Y A A P Q E   90
D S S G D E A E S P S K M K R S K G I H V F K K P S F S K K  120
K E K D F K I K E K P K E E K H K E E K H K E E K H K E K K  150
S K D L T A A D V V K Q W K E K K K K K P I Q E P E V P Q  180
I D V P N L K P I F G I P L A D A V E R T M M Y D G I R L P  210
A V F R E C I D Y V E K Y G M K C E G I Y R V S G I K S K V  240
D E L K A A Y D R E E S T N L E D Y E P N T V A S L L K Q Y  270
L R D L P E N L L T K E L M P R F E E A C G R T T E T E K V  300
Q E F Q R L L K E L P E C N Y L L I S W L I V H M D H V I A  330
K E L E T K M N I Q N I S I V L S P T V Q I S N R V L Y V F  360
F T H V Q E L F G N V V L K Q V M K P L R W S N M A T M P T  390
L P E T Q A G I K E E I R R Q E F L L N C L H R D L Q G G I  420
K D L S K E E R L W E V Q R I L T A L K R K L R E A K R Q E  450
C E T K I A Q E I A S L S K E D V S K E E M N E N E E V I N  480
I L L A Q E N E I L T E Q E E L L A M E Q F L R R Q I A S E  510
K E E I E R L R A E I A E I Q S R Q Q H G R S E T E E Y S S  540
E S E S E S E D E E E L Q I I L E D L Q R Q N E E L E I K N  570
N H L N Q A I H E E R E A I I E L R V Q L R L L Q M Q R A K  600
A E Q Q A Q E D E E P E W R G G A V Q P P R D G V L E P K A  630
A K E Q P K A G K E P A K P S P S R D R K E T S I *
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B Homology with GAP

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BLOCK 1
RLIP76      210  PAVFRECIDYVEKYGMKCEGIYRVSGIKSKVDELKAAYD  248
BCR        1058  PYIVRQCVEEIERRGMEEVGIYRVSGVATDIQALKAAFD 1106
n-Chimaerin 122  PMVVDMCIREIESRGLNSEGLYRVSGFSDLIEDVKMAFD  160
Consensus        P +   C+  +E  G+    G+YR+SG     +  +K A+D

BLOCK 2
RLIP76      259  EPNTVASLLKQYLRDLPENLLTKELMPRFEE  289
BCR        1120  DVEAIAGTLKLYFRELPEPLFTDEFYPNFAE 1150
n-Chimaerin 175  DIKIITGALKLYFRDLPIPLITYDAYPKFIE  205
Consensus        + N +   LK Y R+LP   L T +  P F E

BLOCK 3
RLIP76      310  LPECNYLLISWLIVEMDHVIAKELETKMNIQNISIVLSPTVQIS  353
BCR        1171  LPEANLLTFLFLLDELKRVAEKEAVNKMSLHMLATVFGPTLLRP 1214
n-Chimaerin 226  LPPAHCETLRYLMAELKRVTLHEKENLMSAEMLGIVFGPTLMRS  269
Consensus        LP          +L+ H+  V   E     M+  N+  V  PT+  +
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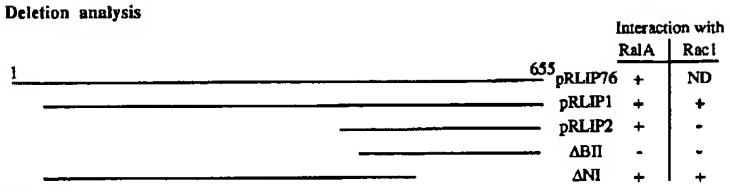
C Deletion analysis



	Interaction with RalA	Rac1
pRLIP76	+	ND
pRLIP1	+	+
pRLIP2	+	-
ΔBII	-	-
ΔNI	+	+

D Domains

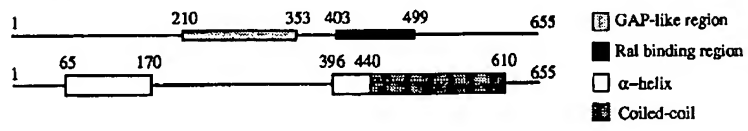


FIG. 4. **Sequence and analysis of RLIP76.** *A*, amino acid sequence of RLIP76. *B*, homology between RLIP76 and GAP domains Both a Blitz homology search program (EMBL) and a BLAST homology search program (NCBI) aligned a region of RLIP76 with domains of proteins that display a CDC42/Rac/Rho GAP activity. The two best scores were obtained with Bcr-GAP region and *n*-chimaerin, and these alignments are shown here. Identical residues are *bold*, conserved residues are indicated by a + on the consensus line. *Blocks 1*, *2*, and *3* refer to three blocks conserved among proteins of this class (22). *C*, deletion analysis. RLIP76 coding region was inserted in plasmid pGAD1318, which allows expression of GAL4AD fusion proteins. From pRLIP1 isolated during the screen, different regions were deleted. For each construct, the junction region was sequenced. These plasmids were tested for their ability to elicit a positive signal in the two-hybrid system in presence of a plasmid expressing a LexA-RalA protein or, when indicated, a LexA-Rac1 protein. *D*, functional regions. Based on the previous analysis (*B* and *C*), two regions can be defined functionally: a GAP region from residue 210 to 353, and a RalA binding region from residue 403 to 499. Compilation of results from three different programs predicting secondary structures (31–33) led to this scheme. The two regions represented by *boxes* are predicted by at least two of the programs to be composed mainly of α-helices. In addition, part of the second region is predicted to be superfolded as a coiled-coil structure (32).

established.

There is one main reading frame (ORF), from base 224 to base 2188, preceded by a correct translation initiation sequence (21). Two short ORFs (13 and 3 codons) are found within the 5′ end of this cDNA but none of them is preceded by a correct translation initiation sequence. A 1664-base pair non-coding sequence is found 3′ to the ORF.

This ORF encodes a protein made of 655 amino acids and of predicted molecular mass 76 kDa that we named RLIP76 (Fig. 4*A*). RLIP1 in plasmid pRLIP1 starts at amino acid 185, and RLIP2 starts at amino acid 403.

Data bank comparison revealed that the region extending

from amino acid 210 to amino acid 353 shares significant homology to regions of proteins bearing a CDC42/Rho/Rac-GAP activity, like Bcr, chimaerins, *Drosophila* rotund, and the Ras-GAP-binding protein p190 (22). Fig. 4*B* shows this striking homology with Bcr and *n*-chimaerin.

Fig. 4*C* gives the results obtained with two-hybrid plasmids expressing different parts of RLIP76. These results allow definition of the maximum size of the region required for RalA binding. Together with secondary structure predictions (Fig. 4*D*), the overall structure of RLIP76 can be depicted schematically as composed of four regions: an N-terminal region where amino acids 65–170 are predicted to be structured in α-helices, the GAP-like region (aa 210–353), the Ral binding region (aa 403–499) predicted to be composed in part of α-helices, and a C-terminal region (aa 499–655). Part of this latter region and part of the Ral binding region (aa 440–610) are predicted to be able to form a coiled-coil structure.

By FISH analysis of 19 R-banded metaphase cells, RLIP1 gene was localized on band 18p11 (25 chromosomes positive on both chromatid out of 38). A minor localization on band 3q26 was also detected (9 out of 38) that might suggest the existence of a related gene (data not shown).

DISCUSSION

We have identified a cDNA encoding a protein, RLIP1, that is able to interact with RalA and RalB, and which has the characteristics of a Ral effector; biochemical data and genetics suggest that RLIP1 binds better to Ral-GTP than to Ral-GDP and that this interaction requires a functional effector domain. According to Northern blot analysis, RLIP1 is ubiquitously (but at low levels) expressed, as are Ral and RalGDS, a Ral activator (3).

Although able to discriminate Ral from other GTPases, RLIP1 also binds to the active form of Rac1. We suppose that the molecular avatar of this binding is a GAP-like region whose absence impairs interaction with Rac1 but not with RalA; domains involved in Rac binding and in Ral binding are physically distinct. We also show that the structural homology with GAP-like regions reflects a functional homology. RLIP1 is able to activate specifically hydrolysis of GTP bound to Rac1 and to CDC42, but not, as expected, to Rap2A.

The whole cDNA was cloned; it encodes a 76-kDa protein, RLIP76, able to bind to RalA as based on a two-hybrid assay.

These findings raise several questions. Our results allow us to conclude that the GAP-like region of RLIP76 displays a *bona fide* GAP activity acting upon CDC42 and Rac1. However, this GAP activity is rather weak when compared to the GAP activity of Bcr tested in parallel. This could be due either to technical problems (we do not know how much of purified RLIP is active), to structural problems (either a larger part or a smaller part of RLIP76 could do better) or to biological constraints (a companion protein might increase this activity). These considerations lead to more questions. What is Ral doing to RLIP76? Is it localizing RLIP76 in the vicinity of its target, as happens to be the case for other GTPases involved in the subcellular localization of certain effectors (23, 24), and/or is it modulating RLIP76-GAP activity?

Ras and Rho pathways are both activated during mitogenic signaling through transmembrane receptors. It is unclear if their activation is sequential or parallel, but they seem to work synergistically (19, 25–29). The Rho pathway, a cascade of GTPases, from CDC42 to Rho passing by Rac, acts upon structures involved in cell shape plasticity. Activation of Ras leads to several cytoplasmic and nuclear phenomena as well as membrane modifications. And Ral proteins are potentially switched to their active form through interaction of activated Ras with Ral activators. We propose that RLIP76 participates in the cross-talk between these GTPase cascades, modulating the state of activity of the CDC42/Rac/Rho pathway in response to Ras activation.

Finally, regions of RLIP76 seem *a priori* not to participate in the above functions. The α-helix-rich regions, especially the coiled-coil region, might be involved in interactions with other proteins. Alternatively, the coiled-coil region might participate in the homodimerization of RLIP76. After Ras and subsequent Ral activation, Ral binding to RLIP76 could separate the monomers and render the GAP catalytic region accessible to its target.

It will be of great interest in future RLIP studies to analyze the regulation and interplay of the various separate functional domains.

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Identification and Characterization of Ral-Binding Protein 1, a Potential Downstream Target of Ral GTPases

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Ral proteins constitute a distinct family of Ras-related GTPases. Although similar to Ras in amino acid sequence, Ral proteins are activated by a unique nucleotide exchange factor and inactivated by a distinct GTPase-activating protein. Unlike Ras, they fail to promote transformed foci when activated versions are expressed in cells. To identify downstream targets that might mediate a Ral-specific function, we used a *Saccharomyces cerevisiae*-based interaction assay to clone a novel cDNA that encodes a Ral-binding protein (RalBP1). RalBP1 binds specifically to the active GTP-bound form of RalA and not to a mutant Ral with a point mutation in its putative effector domain. In addition to a Ral-binding domain, RalBP1 also contains a Rho–GTPase-activating protein domain that interacts preferentially with Rho family member CDC42. Since CDC42 has been implicated in bud site selection in *S. cerevisiae* and filopodium formation in mammalian cells, Ral may function to modulate the actin cytoskeleton through its interactions with RalBP1.

Two closely related Ral proteins (RalA and RalB; 85% identical) constitute a distinct family within the Ras superfamily of GTPases (5). They are among the closest relatives of Ras proteins, sharing 58% sequence identity, and have similar overall structural features (15). For example, their affinities for guanine nucleotides and intrinsic GTPase activities are comparable. Moreover, mutations in Ral that are comparable to oncogenic mutations in Ras also suppress the intrinsic GTPase activity in Ral and make the protein resistant to its GTPase-activating protein (GAP), Ral-GAP (10). Finally, both Ras and Ral are prenylated at the carboxy-terminal CAAX box that targets them to the membrane fraction of cells (21).

Ras and Ral are distinguishable in many respects, however. Unlike Ras, Ral does not produce transformed foci of cells when it is locked in its active GTP state (13). This difference is likely due to the fact that the effector domain of Ral is distinct from that of Ras. This 10- to 15-amino-acid (aa) (aa 26 to 45 in Ras) region present in Ras family members plays an important role in interactions with downstream targets and GAPs. Ras and Ral are also regulated by distinct GAPs (10) and guanine nucleotide exchange factors that promote the replacement of bound GDP with GTP on these GTPases (12). Interestingly, the Ral exchange factor, Ral-GDS, has been shown to bind preferentially to the active forms of Ras, Rap1A, and R-Ras in the *Saccharomyces cerevisiae* two-hybrid system and in vitro (17, 20, 34). This raises the possibility that Ral activation constitutes a distinct downstream pathway from Ras.

The localization of Ral in cells is also different from that of Ras. Whereas Ras is found almost exclusively on the internal face of the plasma membrane, Ral has a more diverse distribution. Ral can be found in plasma membrane fractions, but it is present primarily in cytoplasmic vesicles, including clathrin-coated vesicles and secretory vesicles (2, 9, 36).

To understand the role of Ral function in cells, we used the yeast two-hybrid system to screen for proteins that can bind preferentially to the active GTP-bound form of RalA. Here we describe some properties of one such protein, termed Ral-binding protein 1 (RalBP1).

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MATERIALS AND METHODS

Cell culture and transient expression. COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% iron-enriched calf serum (Hyclone) at 37°C in 5% CO_2. Cells were plated at a density of ~5×10^5/60-mm-diameter plate 1 day before transfection. Adenovirus major late promoter-based expression vector pMT3 (35) was used for transient expression of the cDNAs in COS-7 cells by the DEAE-dextran method.

Interaction cloning with the yeast two-hybrid system. For two-hybrid screening, ralA (72L, 203S) was subcloned into pAS-CYH2 (Trp marker) (8). This vector directs the expression of a fusion protein between the DNA-binding domain of GAL4 and the entire RalA protein. A rat brain cDNA library, cloned into pACT (4) (kindly provided by I. Macara, University of Vermont), contained a total of 5×10^6 primary recombinants with an average insert size of 1.5 kb. *S. cerevisiae* Y190 (*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL-lacZ LYS2::GAL-HIS3 cyh*^{r}), expressing the GAL4 DNA-binding domain–Ral fusion protein, was transformed with the pACT library (Leu marker) with salmon sperm DNA as the carrier, and 2.3×10^6 primary transformants were selected for growth on medium lacking leucine, tryptophan, and histidine and containing 30 mM 3-aminotriazole. The plates were incubated at 30°C for 3 days. Surviving yeast colonies were transferred to nitrocellulose and laid onto minimal medium plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and screened for expression of β-galactosidase by incubation at 30°C for 1 to 2 days. Blue coloration of a colony was indicative of a positive interaction. Of the 200 His^+ colonies, 15 were also $LacZ^+$. The 15 His^+ $LacZ^+$ colonies were rescued from the plate and grown in selective medium. Plasmid DNA was recovered and introduced by electroporation into leucine-deficient *Escherichia coli* KC8. Transformants were plated on minimal medium lacking leucine so that only transformants carrying the library plasmid grew.

Positives were tested for target specificity by retransformation into reporter strain Y190 alone or in conjunction with the Ral-Gal4 DNA-binding domain fusion or with different Gal4 DNA-binding domain fusions. Only library plasmids that did not activate marker expression in the presence of SNF1, Ras, or Ral49N were analyzed further.

Plasmid construction. pASCYH-Ral72L,203S was constructed as follows. The 771-nucleotide *Eco*RI-*Hin*dIII fragment was excised from plasmid ptacRal72L (5). *Bam*HI linkers were added, and the resulting fragment was subcloned into the *Bam*HI site of pGEX3X. The Cys at position 203 was changed to a Ser (C203S), and a 3′ *Sal*I site was introduced by PCR. A *Bam*HI-*Sal*I RalA fragment was then cloned into vector pASCYH. An effector domain mutation was created by using overlap PCR (19) to change the Asp at position 49 to an Asn (D49N). pASCYH-Ras61L186C was constructed as follows. pXCR61L (14) was used as the template for site-directed PCR mutagenesis, which changed Cys-186 to a Ser (C186S) and generated both 5′ and 3′ *Bam*HI sites. The resulting product was excised by *Bam*HI digestion and subcloned into vector pASCYH.

Isolation of the complete RalBP1 cDNA and sequencing. Additional rat RalBP1 cDNAs were isolated from an oligo(dT)-primed and randomly primed brain library (Clontech) by using the 1.6-kb RalBP1 clone isolated from the two-hybrid system as a probe. The RalBP1 cDNA sequence was determined on both strands by the dideoxy-chain termination technique with multiple cDNA clones and subclones.

Northern (RNA) blot analysis. A multiple rat tissue poly(A) RNA blot (Clon-

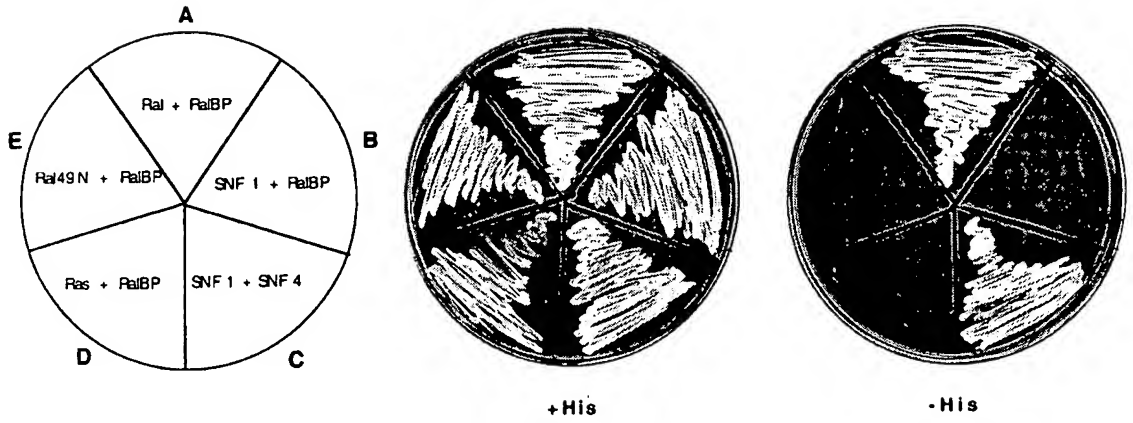

FIG. 1. Specificity of the interaction between RalBP1 and Ral in *S. cerevisiae*. The 1.6-kb cDNA clone (RalBP1) isolated from the two-hybrid screen as a fusion with the Gal4 activation domain was used to cotransform reporter strain *S. cerevisiae* Y190 along with various cDNAs fused to the Gal4 DNA-binding domain. Segments: A, RalA72L; B, SNF1; D, Ras61L; E, Ral49N effector mutant. Cotransformation of the DNA-binding domain of Gal4 fused to SNF1 and the activation domain of Gal4 fused to SNF4 served as a positive control (segment C). To assay for protein interaction-induced growth, cells were streaked on permissive medium (+ His) or on selective medium (− His) containing 3-aminotriazole. The plates were incubated for 2 days and photographed.

tech) was probed for expression of the RalBP1 gene by using randomly primed, ^{32}P-labeled fragments from the 1.6-kb RalBP1 cDNA originally isolated from the expression library.

Expression of Ral and RalBP1. Recombinant Ral and Ral49N were subcloned into pGEX2T and transformed into *E. coli* BL21. The bacteria were induced to express protein with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. The fusion proteins were isolated by affinity chromatography on glutathione-Sepharose, eluted with elution buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 20 mM glutathione), and dialyzed into phosphate-buffered saline. Full-length RalBP1 was inserted as a *Bam*HI-*Eco*RI restriction fragment into an altered version of mammalian expression vector pMT3 (35) that contained a modified Glu epitope (RMEFMPME) 5′ to the cloning site. RalBP1 (positions 86 to 626) was excised from pACT by *Xho*I digestion, and 5′ *Bam*HI and 3′ *Eco*RI cleavage sites were added by PCR. The resulting fragment was subcloned into the same modified pMT3 vector. Glu-tagged RalBP1 was affinity purified from transiently transfected COS-7 cells by using Sepharose A beads coated with anti-Glu immunoglobulin G (kindly provided by S. Powers, Onyx Pharm). The RalBP1 cDNA sequence encoding codons 375 to 626 was amplified by PCR with primers that incorporated *Bam*HI and *Eco*RI restriction sites at the 5′ and 3′ ends, respectively. The amplified products were subcloned into pGEX2T and transformed into *E. coli* BL21. RalBP1 (positions 86 to 415), a subfragment of RalBP1 (positions 86 to 626), was isolated by digestion with *Bam*HI and *Bgl*II. It was subcloned into pGEX-2T and transformed into *E. coli* BL21. Recombinant RalA and RasH proteins were expressed in and purified from *E. coli* as described previously (11).

Binding of Ral to RalBP1 in vitro. In vitro binding assays were performed with GST-RalA or recombinant Ral and either Glu epitope tagged-RalBP1 or glutathione-*S*-transferase (GST)–RalBP1. Glu-tagged RalBP1 (~0.2 μg) bound to Sepharose A beads or RalBP1 fragments (~1.0 μg) bound to glutathione-Sepharose were incubated at 4°C for 3 h with either 0.5 μg of the nucleotide-bound GST-RalA fusion protein or purified RalA, respectively, in 20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–150 mM NaCl–0.5% Nonidet P-40. Ral proteins were preloaded with guanine nucleotides by incubation with 1 mM GDPβS or GTPγS for 20 min at 37°C in 20 mM Tris-HCl (pH 7.5)–1 mM dithiothreitol (DTT)–2 mM EDTA–25 mM NaCl–40 μg of bovine serum albumin per ml. The reactions were stopped by addition of $MgCl_2$ to a final concentration of 10 mM. The beads were washed six times with 1 ml of 20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–20 mM NaCl–0.5% Nonidet P-40 and denatured in Laemmli sample buffer. After separation in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and transfer to nitrocellulose, the Ral protein retained on the beads was detected by immunoblotting with anti-Ral antibody. The anti-RalA antibody was prepared by injection of rabbits with the GST-RalA fusion protein, followed by affinity purification with RalA. Detection was done by chemiluminescence (Amersham) with horseradish peroxidase-conjugated anti-rabbit immunoglobulin. As a control, equivalent amounts of recombinant Ras, bound to either GDPβS or GTPγS, were incubated with GST-RalBP1 (positions 375 to 627) as described above for Ral proteins. Additionally, nucleotide-free Ras was incubated with the catalytic domain of Ras–guanine nucleotide-releasing factor (33) bound to glutathione-Sepharose. Ras proteins retained on the beads were visualized by immunoblotting with anti-Ras antibody (Upstate Biotechnology, Inc.).

Immunoblot analysis of RalBP1. GST-RalBP1 (positions 86 to 415), described above, was purified with glutathione-Sepharose and eluted with 50 mM Tris (pH 8.0)–120 mM NaCl–20 mM glutathione–1 mM DTT and used to immunize rabbits. The resulting antiserum and preimmune serum control were used for immunoblotting at a dilution of 1:100. Rat brains were fractionated into soluble or particulate fractions as follows. The tissue was homogenized in lysis buffer (25 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid [EGTA], 1 mM DTT, 1% aprotinin) with 10 strokes with a Dounce homogenizer. Unbroken cells were removed by three successive centrifugations at 10,000 × *g* for 15 min each time at 4°C. The sample was then separated into soluble and particulate fractions by centrifugation at 100,000 × *g* and 4°C for 60 min. The protein concentration of each fraction was determined by Lowry assay (25). A 75-μg sample of protein from each fraction was denatured in Laemmli sample buffer, separated on an SDS-polyacrylamide gel, and transferred to nitrocellulose. RalBP1 was detected by immunoblotting with an anti-RalBP1 serum described above.

Measurement of GTP hydrolysis rate of p21s. The GTPase activities of RalA, RhoA, Rac1, and CDC42Hs were measured by a nitrocellulose filtration assay as follows. A 2-μg sample of each p21 was incubated with either 10^{-7} M [γ-^{32}P]GTP or 10^{-7} M [α-^{32}P]GTP (650 Ci/mmol; ICN) in the presence of 20 mM Tris-HCl (pH 7.5)–1 mM DTT–5 mM EDTA–40 μg of bovine serum albumin per ml for 10 min at 30°C in a total volume of 50 μl. The exchange reaction was stopped by addition of $MgCl_2$ (10 mM final concentration). An 8-μl sample of the p21–[γ-^{32}P]GTP complex was added to 12 μl of exchange buffer (1 mM GTP, 40 μg of bovine serum albumin per ml, 20 mM Tris-HCl [pH 7.5], 1 mM DTT, 25 mM NaCl, 10 mM $MgCl_2$) in the absence or presence of the RalBP1 GAP domain (positions 86 to 415). The assay was incubated at 30°C for RhoA, RalA, and CDC42Hs and at 15°C for Rac1. Samples of 15 μl were removed from each incubation over a 10-min time course and filtered through nitrocellulose. The filters were washed twice with ice-cold wash buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$), and the amount of [γ-^{32}P]GTP still bound to the filters was measured by scintillation counting. RhoA, Rac, and CDC42Hs were expressed as GST fusion proteins and obtained in purified form after thrombin cleavage.

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned GenBank accession no. U28830.

RESULTS

Identification of a novel Ral-binding protein. A yeast two-hybrid system was used to identify potential downstream targets of the RalA GTPase (8). A Ral–GAL4 DNA-binding domain fusion was constructed in vector pAS-CYH2. A mutant Ral protein with a Gln-to-Leu substitution at position 72 was used. This protein should accumulate in the active GTP-bound state in *S. cerevisiae* because it has decreased intrinsic GTPase activity (15) and fails to respond to Ral-GAP (10). Cys-203 in the CAAX box of RalA was also mutated to Ser to prevent C-terminal geranyl-geranylation. Such a modification is known to target Ral to membrane fractions and may thus prevent nuclear translocation of the fusion protein required in this assay system. Expression of the fusion protein was confirmed

```
1    gaa ttc cgg gcg taa gtg cga gga ggc ggc ggc tgc agc gga gag gac ggt cgc tcg gcg
61   gca ggc act ggt gta atg gat agg taa cag aag acc act tcc ctt cct cct cag ggc ttc
121  agc ATG act gag tgc ttc ctg ccc ccc acc agc agc cct agt gaa cac cgc agg gcc gag
1      M  T  E  C  F  L  P  P  T  S  S  P  S  E  H  R  R  A  E
181  cac ggc agc ggg ctg acc cgg acc ccc agc tcc gag gag atc agc cct aca aaa ttt act
20   H  G  S  G  L  T  R  T  P  S  S  E  E  I  S  P  T  K  F  P
241  gaa ttg tac cgg acg ggt gag cct tca cct ccc cat gac atc ctc cac gag cct cct gat
40   G  L  Y  R  T  G  E  P  S  P  P  H  D  I  L  H  E  P  P  D
301  atc gtg tca gat gat gag aaa gac cat gga aag aaa aaa gga aaa ttt aag aaa aag gag
60   I  V  S  D  D  E  K  D  H  G  K  K  K  G  K  F  K  K  K  E
361  aaa agg act gaa ggc tac gtc gcc ttc cag gaa gac agc tct gga gat gag gct gaa agt
80   K  R  T  E  G  Y  A  A  F  Q  E  D  S  S  G  D  E  A  E  S
421  cct tcc aag atg aag agg tcc aag ggc atc cat gtg ttc aaa aag ccc agc ttc tct aaa
100  P  S  K  M  K  R  S  K  G  I  H  V  F  K  K  P  S  F  S  K
481  aag aag gaa aag gat ttt aaa atc aaa gaa aag ccc aaa gaa gaa aag cac aaa gaa gaa
120  K  K  E  K  D  F  K  I  K  E  K  P  K  E  E  K  H  K  E  E
541  aag cat aaa gag gaa aag cat aaa gaa aag aaa tgt aaa gac ttc acg gcc gcc gat gtt
140  K  H  K  E  E  K  H  K  E  K  K  C  K  D  L  T  A  A  D  V
601  gtt aaa cag tgg aag gaa aag aag aaa aag aag aag cca act cag gag cca gag gtg cct
160  V  K  Q  W  K  E  K  K  K  K  K  K  P  T  Q  E  P  E  V  P
661  cag acg gac gcc ccg agt ctc aga ccc att ttc ggc gct ccc ttc gct gac gca gta gag
180  Q  T  D  A  P  S  L  R  P  I  F  G  A  P  L  A  D  A  V  E
721  agg acc atg atg tac gac ggc atc cgg ctg cca gct gtc ttc cgg gag tgc gtg gac tac
200  R  T  M  M  Y  D  G  I  R  L  P  A  V  F  R  E  C  V  D  Y
781  atg gag aag cac ggc atg aaa tgt gaa ggc atc tac cga gtg tca gga atc aaa tca aag
220  M  E  K  H  G  M  K  C  E  G  I  Y  R  V  S  G  I  K  S  K
841  gta gat gag ctc aaa gca gcc tat gac cga gag gag tct cca aac ttg gaa gaa tat gaa
240  V  D  E  L  K  A  A  Y  D  R  E  E  S  P  N  L  E  E  Y  E
901  cct aac act gta gcc agc ttg ctg aag cag tat ctg cga gac ctt ccc gag aat ttg ctt
260  P  N  T  V  A  S  L  L  K  Q  Y  L  R  D  L  P  E  N  L  L
961  acc aaa gag ctt atg ccc cga ttt gaa gag gct tgt ggg agg acc aca gag gtg gag aaa
280  T  K  E  L  M  P  R  F  E  E  A  C  G  R  T  T  E  V  E  K
1021 gtg cag gag ttc cag cgc ttg ctc agg gaa ctg ccg gag tac aac cat ctt ctg ctc tcc
300  V  Q  E  F  Q  R  L  L  R  E  L  P  E  Y  N  H  L  L  L  S
1081 tgg ctc att gtg cac atg gac cac gtc atc gcg aag gag ctg gaa acc aag atg aac atc
320  W  L  I  V  H  M  D  H  V  I  A  K  E  L  E  T  K  M  N  I
1141 cag aac atc tcc ata gtg ctg agc cca acc gtc cag atc agc aac cgg gtc ctg tat gtg
340  Q  N  I  S  I  V  L  S  P  T  V  Q  I  S  N  R  V  L  Y  V
1201 ctt ttc aca cac gtg caa gag ctc ttt ggc acc gtg ctc ctg aag caa gta acg aga cct
360  L  F  T  H  V  Q  E  L  F  G  T  V  V  L  K  Q  V  T  R  P
1261 ctg cgt tgg tcc aac atg gcc aca atg cct aca ctg cca gag acc caa gca ggc atc aag
380  L  R  W  S  N  M  A  T  M  P  T  L  P  E  T  Q  A  G  I  K
1321 gaa gaa atc agg aga cag gag ttc ctt ttg aac tgt tta cat cga gat ctg cag ggc ggg
400  E  E  I  R  R  Q  E  F  L  L  N  C  L  H  R  D  L  Q  G  G
1381 ata aag gac ttc tct aaa gaa gaa aga tta tgg gaa gta cag agg att ctg act gcc ctc
420  I  K  D  L  S  K  E  E  R  L  W  E  V  Q  R  I  L  T  A  L
1441 aag aga aag ctg agg gaa gct aaa aga caa gag tgt gag acc aag att gca cag gag ata
440  K  R  K  L  R  E  A  K  R  Q  E  C  E  T  K  I  A  Q  E  I
1501 gcc agc ctt tcg aag gag gat gtt tcc aaa gaa gaa acg aac gaa aac gag gag gtc ata
460  A  S  L  S  K  E  D  V  S  K  E  E  T  N  E  N  E  E  V  I
1561 aat atc ctc ctt gcc cag gag aat gag atc ctg act gag cag gag gag ctc ctg gcc atg
480  N  I  L  L  A  Q  E  N  E  I  L  T  E  Q  E  E  L  L  A  M
1621 gag cag ttt ctg cgt cgt cag atc gcc tcg gag aaa gaa gag att gac cgc ctc cga gct
500  E  Q  F  L  R  R  Q  I  A  S  E  K  E  E  I  D  R  L  R  A
1681 gag att gcg gag atc cag agt cgc cag cat ggc cgc agc gag aca gag gag tac tcc tct
520  E  I  A  E  I  Q  S  R  Q  H  G  R  S  E  T  E  E  Y  S  S
1741 gac agc gag agc gag agc gag gat gag gag gag ctg cag atc att ctg gaa gac tta cag
540  D  S  E  S  E  S  E  D  E  E  E  L  Q  I  I  L  E  D  L  Q
1801 agg cag aat gaa gag tta gaa ata aag aac aat cac ttg aac cag gca gtc cat gaa gag
560  R  Q  N  E  E  L  E  I  K  N  N  H  L  N  Q  A  V  H  E  E
1861 cgg gag gcc atc gtt gag ctc cga gtg cag ctc cgg ctg ctc cag atg ctg cga gcc aag
580  R  E  A  I  V  E  L  R  V  Q  L  R  L  L  Q  M  L  R  A  K
1921 tct gag cag cag ctc cag gag gag gag gag ccc gag agg cga ggg ggc aca ggc cca cta
600  S  E  Q  Q  L  Q  E  E  E  E  P  E  R  R  G  G  T  G  P  L
1981 ccc tgt gaa ggt gtc ctc gag gtg aga gca gcc aag gag cag gcg aag ccc tcg ccc agc
620  P  C  E  G  V  L  E  V  R  A  A  K  E  Q  A  K  P  S  P  S
2041 aaa gac cgg aag gag acg ccc atc TGA act gcc ccg gcc ctg tgc atc gta ctg taa gcg
640  K  D  R  K  E  T  P  I  *
```

FIG. 2. Nucleotide and deduced amino acid sequences of the open reading frame encoding RalBP1. The start and stop codons bordering the open reading frame are in capitals, and stop codons preceding the first methionine are underlined. Nucleotide and amino acid sequence numbers are at the left. The sequence homologous to Rho-GAP family members (aa 210 to 352) is underlined, as is a highly basic region predicted to be α-helical.

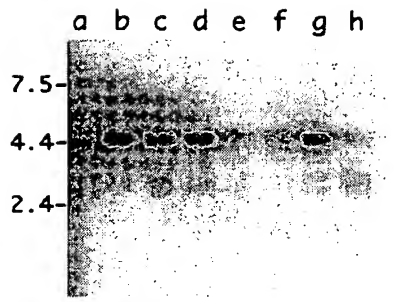

FIG. 3. Tissue distribution of RalBP1 expression. Northern blot analysis of poly(A) mRNAs isolated from a variety of tissues probed with RalBP1 cDNA encoding aa 86 to 626. Lanes: a, heart; b, brain; c, spleen; d, lung; e, liver; f, skeletal muscle; g, kidney; h, testis. The numbers at the left are molecular sizes in kilobases.

by immunoblot analysis of yeast extracts with anti-Ral serum (data not shown). To identify proteins that interact with Ral, the yeast reporter strain, containing pAS-RAL72L,203S, was transformed along with a rat brain cDNA library expressed as fusions to the GAL4 activation domain in pACT (4). From 2.5 $\times$ 10^6 primary transformants, approximately 200 survived the initial histidine selection. Fifteen colonies were also positive for the secondary screen, expression of β-galactosidase. For seven of these clones, histidine prototrophy and β-galactosidase activity depended on the presence of both plasmids. The cDNA library plasmids were transferred into *E. coli* and subjected to DNA sequencing, which revealed that six clones (1.6 kb) were identical and one contained an independent clone of the same gene.

To determine the specificity of this protein-protein interaction, yeast cells were cotransformed with the 1.6-kb partial cDNA for this Ral-binding protein, RalBP1, along with either Ral or one of two control fusion proteins, the SNF1 kinase and constitutively active Ras. As a positive control, we cotransformed SNF1 and SNF4, which are known to interact in the two-hybrid system (Fig. 1). All cotransformants grew well under nonselective conditions in medium containing histidine, but only Ral and RalBP1 and SNF1 and SNF4 grew well under selective conditions in medium lacking histidine. Next, the role of the putative effector domain of Ral (aa 37 to 56) in this binding reaction was evaluated. The analogous region in the mammalian Ras protein (residues 26 to 45) interacts with downstream targets such as Raf, Ras-GAP, and RalGDS (6, 17, 20, 30, 34). Substitution of Asn for Asp at position 38 of Ras blocks its interaction with many of these proteins. Thus, we introduced an equivalent substitution into Ral (49N) and observed that it abolished the protein's interaction with RalBP1 in *S. cerevisiae* (Fig. 1). These results show that RalBP1 interacts specifically with Ral and that this interaction requires a functional effector domain. Thus, RalBP1 may function as a downstream target of Ral.

The 1.6-kb partial cDNA isolated by the two-hybrid system was used as a probe to isolate the entire coding sequence of the gene. A single open reading frame was found that coded for a 647-aa protein with an estimated molecular mass of ~75 kDa (Fig. 2). The cDNA clone was also used to probe Northern blots containing RNAs from a variety of tissues. RalBP1 was found to be expressed in all of the tissues examined as a major transcript of ~4.6 kb (Fig. 3).

Antiserum to RalBP1 was generated by injecting rabbits with a portion of RalBP1 (aa 86 to 415) purified from bacteria as a fusion protein with GST. When the antiserum was used to immunoblot total-cell lysates from rat brain tissue, a predominant band of ~95 kDa was detected. This band likely represented RalBP1 because it was absent when preimmune serum was used (Fig. 4, compare lanes a and b). When brain extracts were fractionated into cytoplasmic and particulate fractions, a similar band was present in both (lanes c and d). The size of this 95-kDa band was significantly greater (~20 kDa) than that predicted from the cDNA. When the full-length cDNA for RalBP1 was expressed transiently in COS-7 cells, it also migrated as a protein of ~95 kDa (Fig. 4, lane e). Furthermore, when the carboxyl-terminal region of RalBP1 (aa 375 to 626; see Fig. 6) was expressed as a fusion protein with GST in *E. coli*, it also migrated to the position of a protein ~20 kDa larger than that predicted (Fig. 4, lane f). Endogenous RalBP1 in COS-7 cells was below the level of detection in this gel (lane g). The discrepancy in apparent size may be due to the abnormal mobility of RalBP1 in SDS-polyacrylamide gels.

Examination of the amino acid sequence of RalBP1 revealed that it contained a domain between residues 210 and 352 that

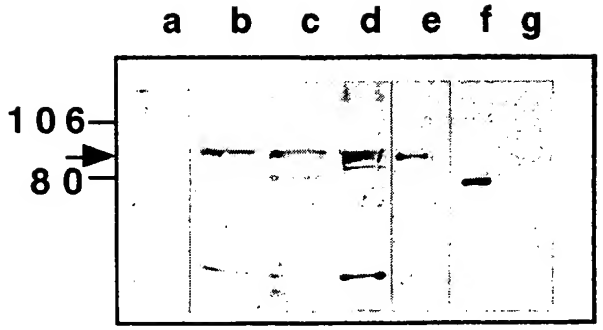

FIG. 4. Immunoblot detection of RalBP1 in cell extracts. Whole-cell lysates (lanes a and b) and cytosolic (lane c) and particulate (lane d) fractions of rat brain tissue were run on SDS-polyacrylamide gels and immunoblotted with antiserum against RalBP1 (positions 86 to 415) or preimmune serum (lane a). Alternatively, whole-cell extracts from COS-7 cells (lane g), from COS-7 cells transiently transfected with RalBP1 (lane e), or from *E. coli* expressing GST-RalBP1 (positions 375 to 626) (lane f) were used. Antibody interaction was detected by a chemiluminescence assay (Amersham) with horseradish peroxidase-conjugated anti-rabbit immunoglobulin. The numbers on the left are molecular sizes in kilodaltons. The arrow indicates full-length RalBP1.

is homologous to a family of GAPs for the Rho family of GTPases (Fig. 2 and 5). The closest homology was with the Rho-GAP domain of BCR (35% identity). Other interesting regions of RalBP1 that warrant further study are an extremely basic region between aa 113 and 172 that contains ~35% lysines (Fig. 2). Secondary-structure analysis predicted that this region forms an α-helix. Another region predicted to be enriched in α-helix (acidic) is between aa 400 and 600. A linear map of the protein is presented in Fig. 6.

Binding of RalBP1 to Ral in vitro. The yeast two-hybrid system indicated that RalBP1 bound specifically to Ral and that binding was dependent upon a functional Ral effector

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                210
      Ralbp1    PAVFRECVDY M.EKHGMKCE GIYRVSGIKS KVDELKAAYD RE
        Bcr     PYIVRQCVEE I.ERRGMEEV GIYRVSGVAT DIQALKAAFD VN
      Nchim     PMVVDMCIRE I.ESRGLNSE GLYRVSGFSD LIEDVKMAFD RD
     B2chim     PMVVDICIRE I.EARGLKSE GLYRVSGFTE HIEDVKMAFD RD
       P190     PIFIERCIEY I.EATGLSTE GIYRVSGNKS EMESLQRQFD QD
      Rhogap    PIVLRETVAY L.QAHALTTE GIFRRSANTQ VVREVQQKYN MG
p122 RhoGAP     PQSIQQAMRY L.RNHCLDQV GLFRKSGVKS RIQALRQMNE SA
      myr5      PIVLEKLLEH V.EMHGLYTE GLYRKSGAAN RTRELRQALQ TD
    Consensus   P  •       •   ••    G••R S•     •  •

      Ralbp1    PNTVASLLKQ YLRDLPENLL TKELMPRFEE ACGRTTEVEK VQE
        Bcr     VNAIAGTLKL YFRELPEPLF TDEFYPNFAE GIALSDPVAK ESC
      Nchim     INIITGALKL YFRDLPIPLI TYDAYPKFIE SAKIMDPDEQ LET
     B2chim     INIITGALKL YFRDLPIPVI TYDTYSKFID AAKISNADER LEA
       P190     VNTVAGAMKS FFSELPDPLV PYSMQIDLVE AHKINDREQK LHA
      Rhogap    LHLPAVILKT FLRELPEPLL TFDLYPHVVG FLNIDESQRV PAT
P122 RhoGAP     V...ADMIKQ YFRDLPEPLM TNKLSETFLQ IYQYVPKDQR LQA
      myr5      IHAITGVLKQ WLRELPEPLM TFAQYGDFLR AVELPEKQEQ LAA
                     •K  •• •LP  ••        •

                                                        352
      Ralbp1    LPEYNHLLLS WLIVHMDHVI AKELETKMNI QNISIVLSPT VQI
        Bcr     LPEANLLTFL FLLDHLKRVA EKEAVNKMSL HNLATVFGPT LLR
      Nchim     LPPAHCETLR YLMAHLKRVT LHEKENLMNA ENLGIVFGPT LMR
     B2chim     LPPAHYETLR YLMIHLKKVT MNEKDNFMNA ENLGIVFGPT LMR
       P190     FPKENHEVFK YVISHLNRVS HNNKVNLMTS ENLSICFWPT LMR
      Rhogap    LPEENYQVLR FLTAFLVQIS AHSDQNKMTN TNLAVVFGPN LLW
P122 RhoGAP     LPDENREVLQ TLLYFLSDVT AAVKENQMTP TNLAVCLAPS LFH
      myr5      LPEANHTSLE RLIFHLVKVA LLEDVNRMSP GALAIIFAPC LLR
                •P    •  •  •  •      M    •  • P  •
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FIG. 5. Alignment of RalBP1 with other Rho-GAP family members with detected GAP activity against Rho GTPases. Sequence comparisons were generated with the PILE-UP program (version 7.2; Wisconsin Genetics Computer Group). Identical amino acids are indicated, and highly conserved residues are marked in a consensus sequence.

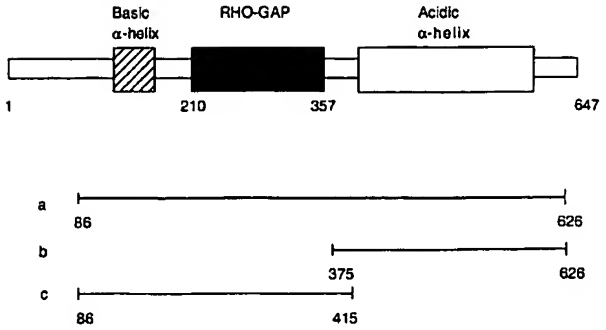

FIG. 6. Linear map of RalBP1 with putative functional domains and fragments expressed in recombinant form. Functional and putative functional domains of RalBP1 are marked. Amino acid sequences of RalBP1 that were expressed in recombinant form are indicated: a, positions 86 to 626; b, positions 375 to 626; c, positions 86 to 415.

domain. These results suggested that RalBP1 could be a downstream target of Ral. If this is so, RalBP1 should bind preferentially to the active, GTP-bound form of Ral. To confirm this hypothesis and to map the region of RalBP1 that binds to Ral, in vitro binding assays were developed.

To measure the nucleotide dependence of binding, the original 1.6-kb cDNA (encoding aa 86 to 626; Fig. 6) of RalBP1 was expressed in COS-7 cells with a Glu epitope added to the N terminus. This form of RalBP1 was immunoprecipitated with anti-Glu antibodies and then incubated with GST-Ral bound to either GTPγS or GDPβS. After washing away of unbound protein, the immune complexes were run on SDS-polyacrylamide gels and immunoblotted with antiserum to RalA (Fig. 7A). As expected, only active GTP-bound Ral bound tightly to RalBP1 (positions 86 to 626). Moreover, the binding of RalBP1 to Ral was blocked by a mutation in the putative effector domain of Ral.

To localize the binding site for Ral on RalBP1, GST fusion constructs containing RalBP1 aa 86 to 626, 86 to 415, and 375 to 626 were generated (Fig. 6). RalBP1 (positions 86 to 626) contained the same amino acid sequences as those used for Fig. 7A. RalBP1 (positions 86 to 415) contained the basic α-helix and the Rho-GAP domain, whereas RalBP1 (positions 375 to 626) contained the remaining carboxyl-terminal sequences of the protein. The encoded proteins were then expressed in bacteria, purified on glutathione beads, and incubated with Ral bound to either GTPγS or GDPβS. After washing, the beads were run on SDS-polyacrylamide gels and immunoblotted with anti-Ral serum (Fig. 7B). Ral-binding activity was found to be associated with the acidic α-helix-rich carboxyl terminus of the protein (lane c) and not with the Rho-GAP domain (lane d). RalBP1 failed to bind Ras complexed to either GTPγS or GDPβS (Fig. 7C, lanes b and c). The Ras protein was functional because in its nucleotide-free bound state, it bound to Ras exchange factor Ras-GRF (Fig. 7C, lane a).

GAP activity of RalBP1. RalBP1 positions 86 to 415 and 375 to 626, described above, were tested for GAP activity against Rho family members CDC42, Rac1, and RhoA, as well as RalA. The proteins were preloaded with [γ-^{32}P]GTP and then incubated with RalBP1 for various lengths of time. [γ-^{32}P]GTP remaining bound to the proteins was measured to assess the rate of GTP hydrolysis (Fig. 8). GTP-binding proteins prebound to [α-^{32}P]GTP were used as negative controls to assess nonspecific losses of GTP. RalBP1 (positions 86 to 415) clearly enhanced the GTP hydrolysis activity of CDC42 (panel A). In

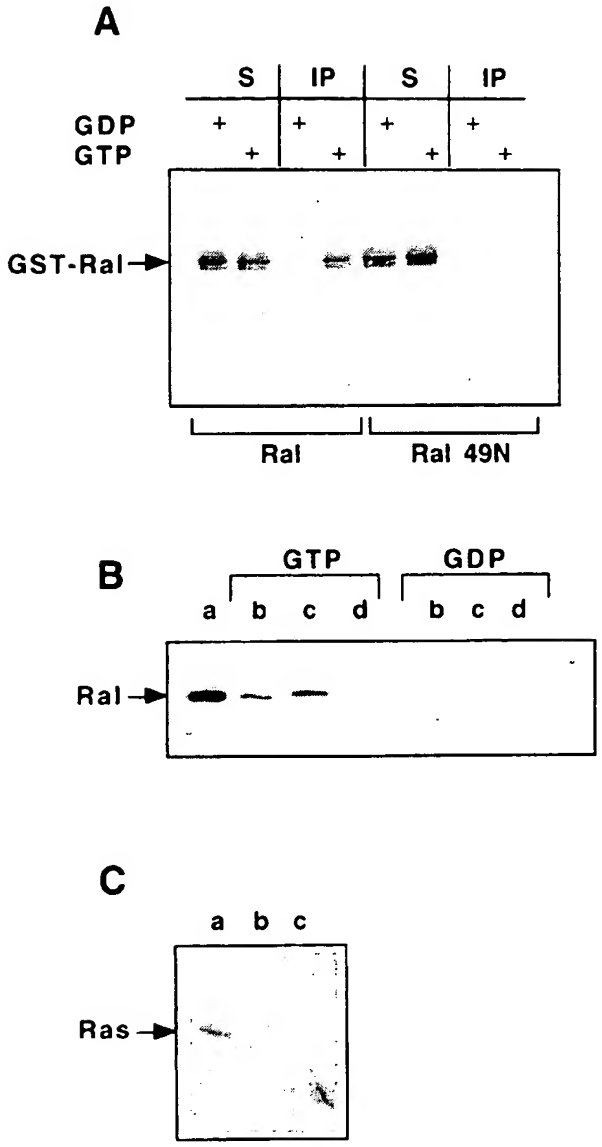

FIG. 7. Ral binding to RalBP1 in vitro. (A) Epitope-tagged RalBP1 (positions 86 to 626) was transiently transfected into COS-7 cells. The protein was then immunoprecipitated from cell lysates and incubated with either GST-RalA or effector mutant GST-RalA49N preloaded with either GTPγS or GDPβS. RalBP1-containing beads were then washed, loaded onto SDS-polyacrylamide gels, and immunoblotted with anti-Ral serum. S, supernatant from washes; IP, washed beads containing RalBP1. (B) GST-RalBP1 positions 86 to 626 (lanes b), 375 to 626 (lanes c), and 86 to 415 (lanes d) were purified from bacterial lysates on glutathione beads and incubated with RalA bound to either GTPγS or GDPβS. The beads were then washed, run on SDS-polyacrylamide gels, and immunoblotted with anti-Ral serum. Recombinant RalA was run as a size standard (lane a). (C) GST-RalBP1 (positions 375 to 626) bound to glutathione beads was incubated with RasH loaded with either GTPγS (lane b) or GDPβS (lane c). The beads were washed, loaded into SDS-polyacrylamide gels, and immunoblotted with anti-Ras serum. As a positive control, nucleotide-free RasH was incubated with GST-GRF. The beads were washed and run on the same gel (lane a) for immunoblotting with anti-Ras serum.

contrast, the GAP domain of RalBP1 had little, if any, effect on Rac1 (panel B) and no observable effect on RhoA (panel C) or RalA (data not shown). RalBP1 (positions 375 to 626), containing the Ral-binding activity, also had no detectable GAP activity on any of these proteins (data not shown), demonstrating that RalBP1 is not likely to be the previously detected Ral-GAP (10).

DISCUSSION

Interest in the function of Ral GTPases has increased recently, with the discovery that Ral GDS, the exchange factor that activates Ral, binds to the active GTP-bound form of Ras in the *S. cerevisiae* two-hybrid system and in vitro (17, 20, 34). Thus, it is possible that activation of Ral represents a distinct downstream pathway from Ras.

To identify proteins that may mediate Ral function in cells, we used the *S. cerevisiae* two-hybrid system. This report describes the cloning and characterization of a novel protein, RalBP1, that has many properties expected of such a downstream target of Ral proteins. First, RalBP1 binds specifically to the activated GTP-bound form of RalA. Second, this binding interaction is inhibited by a mutation (D49N) in the putative effector domain of Ral. In Ras, an analogous Asp-to-Asn mutation is known to block interactions with downstream targets such as Raf. Since RalB differs from RalA in amino acid sequences outside known effector domains, it too likely interacts with RalBP1. Finally, like Ral proteins, RalBP1 displays a ubiquitous tissue expression pattern.

The first clue to the function of RalBP1 came from the observation that in addition to a Ral-binding domain, the protein also contains a Rho-GAP domain. Rho-GAPs are a family of proteins that inactivate Rho GTPases by enhancing their intrinsic GTPase activity (23). By interacting preferentially with the GTP-bound form of Rho proteins, some Rho-GAPs may also function to transmit signals from these Rho proteins to downstream targets. A property common to Rho family members is the ability to influence the actin cytoskeleton (16). For example, Rho proteins mediate growth factor alterations of focal adhesions and actin stress fibers, while Rac proteins link receptors to actin polymerization associated with plasma membrane ruffling (16, 28, 29). Moreover, CDC42 has been implicated in bud site selection in *S. cerevisiae* (1) and filopodium formation in mammalian cells (22, 26). Interestingly, these GTPases may form a cascade of GTPase cycles in which CDC42 leads to Rac activation, which then leads to Rho activation (26).

At least eight functional mammalian Rho-GAPs have been detected. They are a diverse group of proteins with differing specificities toward Rho family members. They also contain a variety of additional signaling domains that may endow them with distinct cellular functions. For example, p190 is a GAP for all three of the best-characterized Rho subfamilies, Rho, Rac, and CDC42 (31). p190 binds to Ras-GAP upon cell stimulation and may help coordinate Ras and Rho signaling pathways. The BCR protein contains a Rho-GAP domain that is functional on Rac and CDC42 but not on Rho (7). BCR also contains a Ser-Thr kinase domain whose function is poorly understood. Rho-GAPs such as N-chimerin and B-chimerin are specific for Rac. Distinguishing features of these GAPs are their regulation by lipids and their tissue-specific expression (7, 24). More recently, a Rho-GAP containing a phospholipase *c*-activating domain has been cloned. It is a GAP for Rho but not Rac (18). Finally, a GAP specific for Rho that contains a myosin domain has been characterized (27). This is of particular interest since it could directly link Rho proteins to actin.

RalBP1 needs to be added to this growing family of regulatory proteins. RalBP1 can be distinguished from these other family members in that it displays the greatest specificity toward CDC42, at least in vitro. Microinjection of CDC42 into

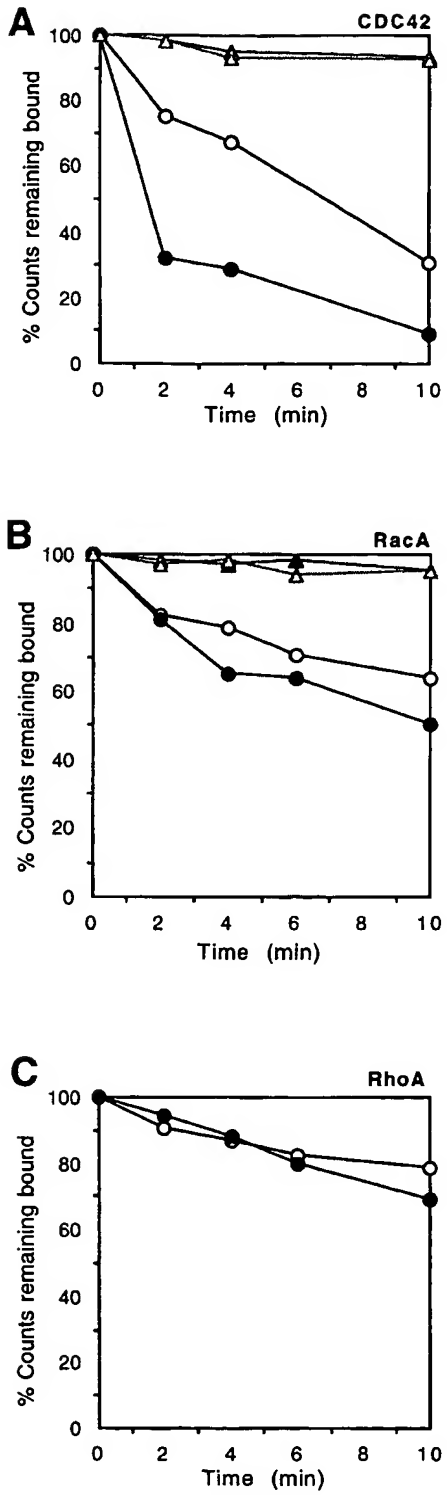

FIG. 8. GAP activity of RalBP1. GST RalBP1 (positions 86 to 415) was incubated with [γ-^{32}P]GTP-CDC42Hs (A), [γ-^{32}P]GTP-Rac1 (B), or [γ-^{32}P] RhoA (C) for the indicated amounts of time. The samples were then passed through nitrocellulose filters, and the amounts of radioactivity remaining associated with CDC42, Rac1, and RhoA were determined by filtering the free radioactivity through nitrocellulose filters. GAP activity is indicated by loss of radioactive counts from the GTPases. As controls, GST-RalBP1 (positions 86 to 415) was incubated with CDC42 and Rac1 bound to [α-^{32}P]GTP. GAP activity should not promote the loss of counts associated with the GTPases labeled in this fashion. Symbols: ○ and ●, [γ-^{32}P]GTP; △ and ▲, [α-^{32}P]GTP. Filled symbols, RalBP1 added; open symbols, no RalBP1 added.

cells has recently been shown to promote actin microspikes and filopodium formation (22, 26). Filopodia are actin-containing structures which have been proposed to have a sensory function in fibroblasts and neural growth cones. Interestingly, a similar phenotype has also been noted in cells stimulated with the neuropeptide bradykinin (22). In fact, inhibition of endogenous CDC42 activation by injection of dominant negative CDC42 blocked these effects of bradykinin. Thus, by binding to a CDC42-GAP, Ral may influence these actin-mediated cellular activities. Since, at least in some cells, CDC42 may regulate the activity of Rac and Rho proteins (26), RalBP1 may also indirectly influence plasma membrane ruffling and actin stress fiber formation.

How might Ral binding to RalBP1 influence CDC42 function in cells? Ral could attract RalBP1 to membrane compartments where Ral is known to reside. This would lead to local inactivation of CDC42. Ral has been detected in plasma membrane fractions. However, most of the protein was found in intracellular vesicles, including clathrin-coated vesicles and secretory vesicles, suggesting that Ral is rapidly internalized from the cell surface (2, 9, 36). Thus, Ral could target RalBP1 to the plasma membrane to influence CDC42 function in filopodium formation. Alternatively, Ral could target RalBP1 to intracellular vesicles, where CDC42 could theoretically influence the interaction between vesicles and the cytoskeleton. This model is consistent with the existence of many GAPs in cells that have the potential to alter CDC42 function at discreet cellular locations. This mechanism is also consistent with the emerging theme that Ras family members function as regulatable localizing devices for other signaling molecules.

By binding selectively to the active GTP-bound form of CDC42, RalBP1 could also function to transmit signals downstream from this GTPase. A body of evidence supports such a role for Ras-GAP (3). Again, one would predict that this is a localized effect at the site of Ral-RalBP1 interactions. Finally, Ral binding might inhibit the GAP activity of RalBP1. If RalBP1 is normally in the vicinity of its target CDC42, activation of Ral could then lead to the activation of CDC42.

RalBP1 may mediate additional Ral functions through activities encoded by other regions of RalBP1, such as the basic α-helix near its amino terminus. Ral also is known to interact with a distinct Ral-GAP that could be responsible for transmitting other Ral-induced cellular effects (10).

It is becoming clear that although members of the Ras superfamily display distinct functions in cells, they form a complicated network of GTPase cycles. For example, the Ras GTPase cycle may influence the Ral GTPase cycle (17, 20, 34), and here we show that the Ral GTPase cycle has the potential to influence the GTPase cycle of CDC42. Ras has already been connected to Rho family members through the Ras-GAP binding partner, p190 Rho-GAP (32). In addition, Ras nucleotide exchange factors Ras-GRF and SOS have putative exchange domains for Rho family members (12). Finally, within the Rho family, new data have revealed multiple interactions between the activity cycles of Rho, Rac, and CDC42 proteins (22, 26). Undoubtedly, these complicated cascades are necessary for these GTPases to help coordinate the complex phenotypes displayed by eukaryotic organisms.

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